# A Fish Two Generation Toxicity Test Detailed Review Paper

Endocrine Disruptor Methods
Validation Subcommittee
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# Detailed Review Paper: A Fish Two Generation Toxicity Test Detailed Review Paper

**WORK PERFORMED BY:**

Battelle
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**and**

***SpringbornSmithers Laboratories LLC***

**On behalf of the United States Environmental Protection Agency EPA CONTRACT NUMBER 68-W-01-023**

# METHODS USED IN THIS ANALYSIS

- On-line Literature Search
  - "Dialog" On-Line search with database Biosis Previews Aquatic Science and Fisheries Abstracts
  - Endocrine disruptor screening methods for fathead minnow, zebrafish, medaka, sheepshead minnow
  - Key Words "estrogen* or testosteron* or endocrin* or antiandrogen* or androgen* or hormon* or thyroxin* or * thyroid * method, protocol etc…
  - 7453 records were refined down to 601 records

# METHODS USED IN THIS ANALYSIS Cont.

Interviews With The Following Experts

Tom Hutchinson

Reynaldo Patino

Taisen Iguchi

Alf Lundgren

Dan G. Cyr

Nancy Denslow

# METHODS USED IN THIS ANALYSIS Cont.

External/Internal Peer Review

- Dr. Dave Hinton – Duke Unv. USA
- Dr. John Sumpter – Brunell Unv. UK
- Dr. Gary Thorgaard - Univ. of Wash. USA
- EPA Technical Experts

# OVERVIEW AND SCIENTIFIC BASIS

- Evidence exists that EDCs affect sexual differentiation, development, and reproduction in fish
- Fish life cycle test methods have been standardized for decades
- Existing methods do not assess transgenerational effects and lack relevant biochemical, morphological, and behavioral endpoints
- The proposed Fish Two Generation Test addresses these EDC relevant omissions

# Test Species

- Fathead minnow (*Pimephales promelas*)
- Medaka (*Oryzias latipes*)
- Zebrafish (*Danio rerio*)
- Sheepshead minnow (*Cyprinodon variegatus)*

  - small size at maturity
  - ease of culture
  - maintenance costs
  - asynchronous spawners

# Fathead Minnow
## Family Cyprinidae

- 35 to 75 mm length
- Extensive aquatic toxicity in USA
- generation time about 4 months
- sexually dimorphic
- females produce 50 to 250 embryos per spawn

# Fathead Minnow

## Strengths

- Large enough to collect individual blood plasma samples
- Distinct secondary sex characteristics in both sexes
- Large historical regulatory database
- Many laboratories are familiar with culture and testing
- Spawn on a substrate
- High fertilization rate
- Indigenous to North America

## Weaknesses

- Relatively long life cycle
- Relatively high variability in fecundity
- Relative size of the fish requires more space for culture and testing
- Intersex condition is less frequently observed compared to other fishes.
- Genome poorly characterized

# Medaka
## Family Adrianichthyidae

- indigenous to Japan, Taiwan, and southeastern Asia
- Generation interval of 2 to 3 months
- sexually dimorphic
- 25 mm to 50 mm length
- females produce 10 to 30 eggs per spawn
- estimated to be over 500 cultivated strains
  - Genetically Engineered / Inbred Strains in Toxicity Testing

# Medaka

## Strengths

- Relatively short life cycle
- Relatively small fish, making culture and testing possible in smaller space
- Female sex determined during embryo stage vs. male sex determined after hatch
- Sex-linked color strain

## Weaknesses

- Smaller size reduces individual blood sample volumes compared to fathead minnow
- Less distinctive secondary sex characteristics
- Regulatory data base less extensive compared to fathead minnow.
- Limited use in short-term tests in the U.S.A.

# Zebrafish
## Family Cyprinidae

- Native to East India and Burma
- 4 cm to 5 cm in length
- Extensive aquatic toxicity in Europe
- Difficult to sex zebrafish
- Sexual maturity in 10 to 12 weeks
- 150 to 400 eggs per female
- Development of transgenic zebrafish

# Zebrafish

## Strengths

- Short life cycle
- Small fish, making culture and testing possible in smaller spaces
- Male fish go through a hermaphroditic phase as juveniles
- Widely used in other medical and genetic research
- Frequently used in Europe for regulatory purposes
- Transgenic fish increasingly available
- Anticipated that entire genome will be sequenced soon.

## Weaknesses

- Small size makes individual blood plasma samples not likely
- Minimal secondary sex characteristics
- Limited US regulatory data base
- Limited testing experience in the US

# Sheepshead Minnow
## Family Cyprinodonitidae

- Native to Atlantic and Gulf of Mexico estuaries
- 35 mm to 50 mm length
- Tolerates wide ranges in temperature (0 to 40°C) and salinity (0.1 ppt to 149 ppt)
- Sexually dimorphic
- Sexual maturity in 60 days
- Low variability in fecundity
- Large historical regulatory database

# Sheepshead Minnow

## Strengths

- Very short life cycle (<60days to sexual maturity), seawater costs may be offset by shorter exposure times for testing
- Males large enough for individual blood plasma samples
- Distinct sexual dimorphism
- Relatively low variability in fecundity
- Relatively large historical regulatory database
- Many laboratories are familiar with culture and testing
- Relatively small fish making culture and testing possible in smaller space

## Weaknesses

- Estuarine/marine species, salinity of 15 to 30 ppt recommended, however, lower salinity may be possible (5 ppt)
- Culture requires a large number of females to produce enough eggs in a 24-hr period to initiate a life-cycle test
- Limited information on reproductive endocrinology
- Small size makes individual blood plasma samples not likely

# Routes of Administration of Chemical Exposure

- Aqueous

- Dietary exposures
- Direct injection techniques
  - Intravascular
  - intraperitoneal

# Measurement Endpoints

- Growth and Morphological Alterations
  - Gonadosomatic Index
  - Histology Techniques
  - Sexual Differentiation
  - Secondary Sex Characteristics
- Measures of Reproductive Performance
  - Fecundity
  - Gamete Viability
  - Changes in Spawning Behavior
- Biochemical Measures
  - Vitellogenin Induction
  - Tissue Steroid Concentrations
  - Thyroid hormones

# MEASUREMENT OF BIOCHEMICAL ENDPOINTS

- Sex Steroids in Tissues Estrogens/Androgens/Progestins
  - Radioimmunoassay (RIA)
  - Enzyme-linked Immunosorbent Assay (ELISA)
  - Liquid/Gas Chromatography with Mass Selective Detection (LC/GC-MS)

# Measurement of Vitellogenin

- Indirect Quantification of Vitellogenin Protein
  - Alkaline-labile Phosphate Assay
- Direct Quantification of Vitellogenin Protein
  - RIA
  - Enzyme-linked Immunosorbent Assay
    - Antibody-capture
    - Antigen-capture
  - Direct Enzyme-linked Immunosorbent Assay
  - Sandwich Enzyme-linked Immunosorbent Assay
- Quantifying Vitellogenin mRNA
  - Ribonuclease Protection Assay (RPA)
  - Quantitative Reverse Transcription-Polymerase Chain Reaction (QRT-PCR)
- Mass spectrometry (MS)

# CANDIDATE PROTOCOLS

1) **Partial Life-Cycle Test**
   {Adult (P) to Juvenile (F1)}

2) **Full Life-Cycle Test**
   {Egg (P) to Juvenile (F1)}

3) **Multi-Generation Test**
   {Egg (P) to Juvenile (F2)}

4) **Two Generation Test**
   {Adult (P) to Juvenile (F2)}

# Partial Life-Cycle Test {Adult (P) to Juvenile (F1)}

A partial life-cycle toxicity test, which exposes P adult, sexually mature fish and the early life cycle of F1 fish, ( can be used to estimate the NOEC)

A pre-exposure reproductive evaluation is conducted on the P fish.

Biological endpoints evaluated:

- P pre-exposure, secondary sexual characteristics and fecundity/reproduction (e.g., eggs/female)
- P post-exposure, survival, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), GSI, histopathology, and protein and sex steroid biomarkers (e.g., VTG)
- F1 hatching success, survival, growth (length and weight)

# Full Life-Cycle Test {Egg (P) to Juvenile (F1)}

A full life cycle test developed (Benoit 1981- fathead minnows ) and (Hansen et al. 1978 - sheepshead minnow).

Initiated with fertilized eggs (P) and the fish are continuously exposed through reproductive maturity, followed assessment of the early development of the F1 generation.

Biological endpoints evaluated:

- P embryo time-to-hatch, hatching success, larval survival and length, weight of thinned fish, survival, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), growth.
- F1 hatching success, survival, growth (length and weight)

# Multi-Generation Test {Egg (P) to Juvenile (F2)}

Multi-generation toxicity test, exposes all life-stages of two generations of fish

Test is initiated with eggs and two full generations of fish are exposed during the test (can estimate the NOEC)

Biological endpoints evaluated:

C P and F1 hatching success, survival, growth (length and weight), time-to-maturity, sex ratio, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), and protein and sex steroid biomarkers (e.g., VTG).

- F2 hatching success, survival and growth.

# Two Generation Test {Adult (P) to Juvenile (F2)}

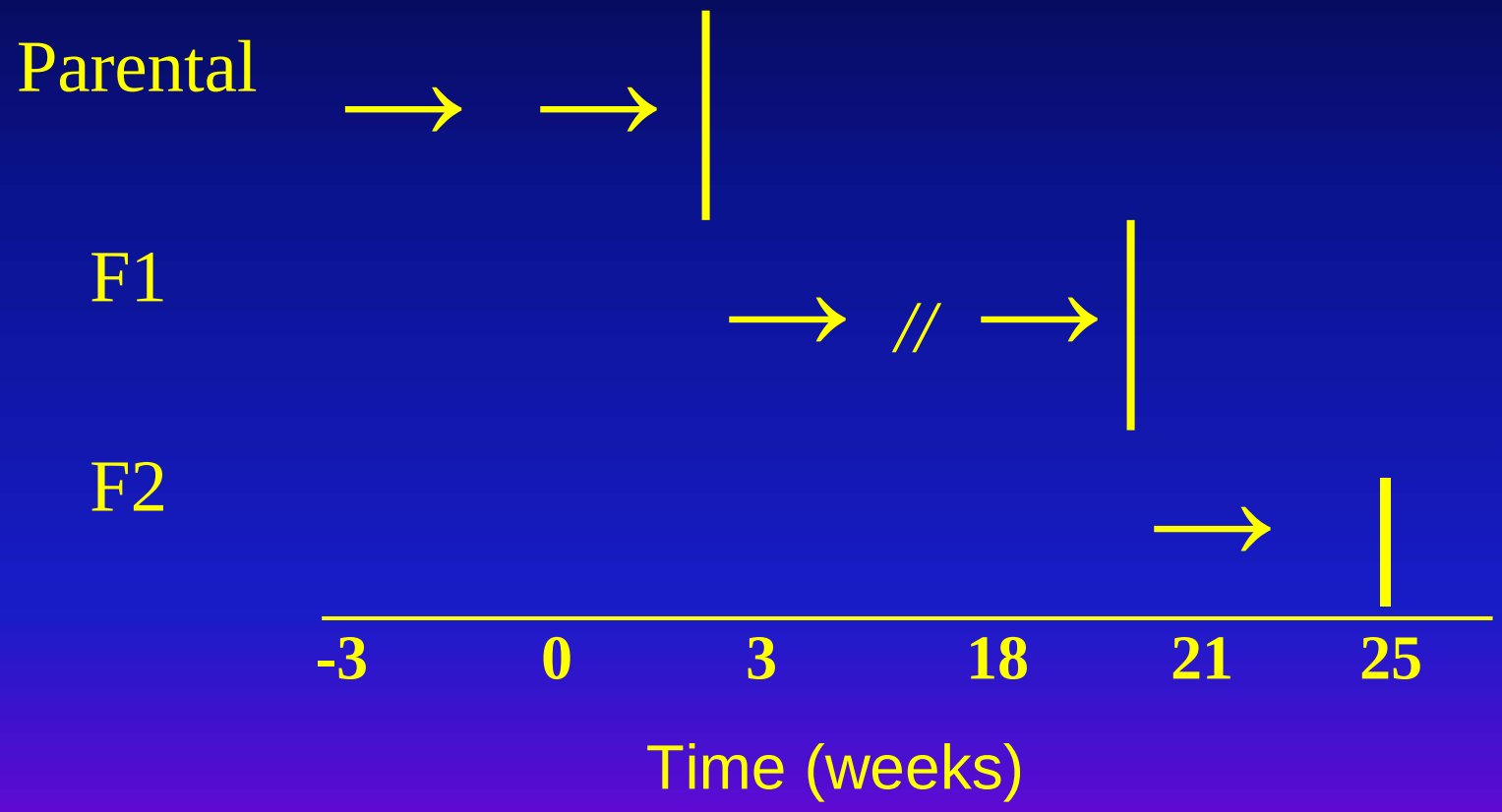
Two generation life-cycle toxicity test, exposes the adult P, full F1generation,and measures F2 viability

Variables including the time-line of the test, the number of fish required in the test, and obtaining endpoints such as Vtg plasma levels will be associated with the different species.

Biological endpoints evaluated:

- P Survival, secondary sex characteristics, reproductive behavior, spawning activity, fecundity, fertilization success
- F1 hatching success, survival, growth (length and weight), time-to-maturity, sex ratio, secondary sexual characteristics, fecundity/reproduction (e.g., eggs/female), and protein and sex steroid biomarkers (e.g., VTG).
- F2 hatching success, survival and growth.

# Timeline forTwo-Generation Protocol with the Fathead Minnow



# Significant Data Gaps

- Male-specific effects of estrogen agonists other than VTG induction.
- The effects of anti-estrogens
- The effects of androgen agonists and antagonists
- Baseline data for thyroid hormone levels in test species.
- The effects of thyroid hormone agonists (or thyroid stimulation) on reproduction.
- Published methods of sexual differentiation for fathead and sheepshead minnows

# IMPLEMENTATION CONSIDERATIONS

- Pre-validation studies following the ICCVAM validation process
  - Recommend evaluating the increased sensitivity of a two-generation design over the existing fish full life-cycle standard practice
  - Recommend determining and optimizing specific two generation protocol variables for the candidate species
  - Recommend demonstration of sensitivity, reliability and reproducibility for each species in the recommended protocol
- Validation of the study design through interlaboratory comparisons